Characterization of an Acyclovir-Resistant Herpes Simplex Virus Type 2 Strain Isolated from a Premature Neonate

Ronda J. Oram,1 Daniel Marcellino,2 Daniel Strauss,2 Erik Gustafson,3 Christine L. Talarico,4 Adrienne K. Root,4 Prem L. Sharma,1,a Ken Thompson,1 Joyce D. Fingeroth,3 Clyde Crumpacker,3 and Betsy C. Herold2

Acyclovir resistance is not a recognized problem among neonates with perinatal herpes simplex virus (HSV) infection. A premature newborn with neurocutaneous HSV infection was treated for 21 days with acyclovir. Disseminated disease recurred 8 days later. A recurrent isolate was resistant to acyclovir and lacked thymidine kinase activity on the basis of a frameshift mutation in the thymidine kinase (tk) gene. Compared with the sensitive isolate obtained during primary infection, replication of the resistant isolate was reduced on primary and permanent cells and even further impaired on cells deleted for cellular tk. The resistant isolate lacked virulence in a murine model of genital infection. Acyclovir-resistant HSV-2 mutants can develop rapidly in neonatal infection and cause clinically significant disease, despite decreased replication in vitro and attenuated virulence in an animal model.

Patient and Methods

Case report. A 708-g boy was born at 26 weeks gestation to a 19-year-old woman who was seronegative for human immunodeficiency virus. The mother had no history of genital herpes infections or treatment with acyclovir. On day 6, the infant developed apnea, bradycardia, neutropenia (absolute neutrophil count, 459), thrombocytopenia (26,000/mm3 platelets), and seizures. Therapy was initiated with vancomycin, gentamicin, and amphotericin. Bacterial cultures were sterile. On day 10, vesicular lesions developed on the face. Cultures from the vesicles and the oropharynx grew HSV-2. No virus was isolated from an endotracheal tube culture. Liver transaminase levels were normal, as were cerebrospinal fluid (CSF) parameters, but HSV DNA was detected in the CSF by polymerase chain reaction (PCR). The results of an electroencephalogram were normal for age. Ophthalmologic examination revealed bilateral herpetic stromal keratitis. The neonate was treated with intravenous acyclovir (20 mg/kg/dose) for 21 days and trifluridine 1% ophthalmic drops. HSV DNA remained detectable by PCR analysis of a CSF sample obtained 48 h after completion of acyclovir therapy.

New skin vesicles developed 8 days after completion of acyclovir therapy (day 39). Acyclovir (20 mg/kg/dose) was re instituted. The second course was complicated by disseminated intravascular coagulation, pneumatosis intestinalis, bilateral pulmonary disease, and seizures. An endotracheal tube culture on day 42 yielded HSV-2. Findings from a repeat evaluation of CSF were as follows: red blood cells, 1388/μL; white blood cells, 12/μL; glucose, 41 mg/dL; and protein, 157 mg/dL. PCR analysis of CSF remained positive for HSV. Multiple cultures during the second course of acyclovir were negative for bacterial pathogens. A repeat endotracheal tube culture for HSV obtained on day 53 did not grow. On day 59, the second 21-day course of acyclovir was completed. Blood cultures grew Candida albicans and remained positive despite treatment with amphotericin. The child died on day 65. A request for an autopsy was denied.

Cells and virus isolates. Vero, CaSki, baby hamster kidney (BHK), and human osteosarcoma 143 TK- cells (all from American Type Culture Collection, Rockville, MD) were cultured as described elsewhere [3–5]. BHK(TK-) clone 100 is derived from the
sequences used for amplification for the coding strand were 5′-TCTGACG-3′ and 5′-CCGGCGTATGGGACACAC-3′; noncoding sequences were 5′-TATATTACGAC-TGACATAGGGAGAGTGGCGCAGCTGCTTCAT-3′ and 5′-biotin-CCGGCGTATGGGACACAC-3′. The samples were separated by use of streptavidin-coated magnetic beads (Dynabeads; Dynal, Oslo). Solid-phase single-stranded DNA sequencing was performed on both strands by use of Taq Dyedeoxy Big Dye Terminator cycle sequencing chemistry with AmpliTaq fluorescence-sequencing polymerase (Applied Biosystems, Foster City, CA) in accordance with the manufacturer’s protocols. Primers were based on the same GenBank sequence and used 12 primers for the coding strand and 7 primers for the noncoding strand. Protein and nucleotide (nt) sequences were analyzed with Lasergene biocomputing software (DNASTAR, Madison, WI) and compared with HSV-2 MS2 and 333.

In vitro growth studies. Vero, CaSki, primary human cervical, BHK, and BHK(TK–) clone 100 cells were infected with plaque-purified DT1 or DT2 at an MOI of 5 for one-step growth or 0.01 for multistep growth, as described elsewhere [5]. HSV-2 333 and 333/TK– served as controls.

Infection of mice. Four- to 6-week-old Balb/c female mice (Jackson Laboratory, Bar Harbor, ME) pretreated with 2 mg of medroxyprogesterone (Upjohn Pharmacia, Kalamazoo, MI) were anesthetized with sodium pentobarbital and inoculated intravaginally with 10 μL of a suspension containing 4.0 log10 pfu HSV-2 333, 333/TK–, DT1, or DT2 (6 mice/virus). The mice were examined daily through day 14 for symptomatic infection that included perineal hair loss and erythema, hind limb paralysis, and mortality. Animals were sacrificed if they exhibited hind limb paralysis or appeared moribund.

Statistics. Incidence data were compared by Mann-Whitney U test or Fisher’s exact test. All comparisons were 2-tailed.

Results

Mutation in tk gene causes acyclovir resistance in the clinical isolate. Because the neonate developed a recurrent disseminated HSV-2 infection shortly after completing acyclovir therapy, the emergence of resistance was considered. DT1 was acyclovir sensitive with an ED50 of 1.0 μg/mL, whereas DT2 was

Figure 1. Thymidine kinase phenotype and genotype of HSV-2 clinical isolates. A. Thymidine phosphorylation (counts per minute [CPM] vs. time) measured by disk assay for each isolate. B. Sequence from nt 921–940 is shown for plaque-purified DT1 and DT2 and laboratory strains HSV-2 333 and MS2.

References

2. Behring Diagnostics, Cupertino, CA.
3. Jackson Laboratory, Bar Harbor, ME.
4. Syva Microtrak HSV1/HSV2; Behring Diagnostics, Cupertino, CA.
5. Beckman, Fullerton, CA.
7. Jackson Laboratory, Bar Harbor, ME.
8. Syva Microtrak HSV1/HSV2; Behring Diagnostics, Cupertino, CA.
9. Syva Microtrak HSV1/HSV2; Behring Diagnostics, Cupertino, CA.
10. Research Triangle Park, NC.
11. Behring Diagnostics, Cupertino, CA.
12. Beckman, Fullerton, CA.
13. Jackson Laboratory, Bar Harbor, ME.
14. Beckman, Fullerton, CA.
Figure 2. Replication kinetics of DT1 and DT2 on primary human cervical cells and BHK cells. A, Multistep growth on primary human cervical cells. B and C, Single- and multistep growth on BHK cells, respectively. In all experiments, cells were infected at MOI of 5 for single-step or 0.01 for multistep growth with DT1 or DT2 (based on titer on Vero cells). At times shown, supernatants and pellets were harvested and titered on Vero cells. Each point represents mean of duplicate determinants from representative experiment. Range of findings was too small to be shown as error bars on plots.

highly resistant (ED$_{50}$ > 50 µg/mL) by plaque-reduction assay (data not shown). No subsequent isolates were obtained. Resistance was mediated by loss of TK activity due to deletion of thymidine at nt 927 of the tk gene. This mutation resulted in a change in amino acid (aa) 309 from phenylalanine to leucine and early termination at nt 1044 or aa 348 (figure 1). The 2 HSV-2 isolates had identical restriction-endonuclease patterns for 4 restriction endonucleases (BamHI, EcoRI, HindIII, and KpnI), suggesting that the recurrent isolate emerged from the primary isolate, even though the 2 viruses were obtained at different times and from different sites (data not shown).

Reduced growth kinetics in acyclovir-resistant isolate. We compared single- and multistep replication kinetics of DT1 and DT2 in several cell types. Although growth kinetics were similar, virus yield was reduced for DT2, particularly in multistep growth studies, which suggests impaired cell-cell spread (figure 2A). Virus yield was also reduced for DT2 compared with DT1 on CaSki and Vero cells and for 333/TK$^-$ compared with HSV-2(333) on all cell types (data not shown). To determine whether cellular TK contributes to viral replication, we compared growth on TK$^+$ and TK$^-$ cells (figure 2B, 2C). In single-step growth experiments, viral yields were reduced for both DT1 and DT2 on TK$^-$ cells compared with TK$^+$ cells, which suggests a role for cellular TK in supporting viral growth. In the multistep growth experiments, DT1 and DT2 exhibited similar growth kinetics and yield on BHK(TK$^+$) cells, which suggests that cellular TK compensated for absent viral TK on this cell line (figure 2C). Again, both viruses exhibited impaired growth on TK$^-$ cells. Yields were lowest when both viral and cellular TK were absent.

Acyclovir-resistant isolate is avirulent in a mouse genital herpes model. All 12 animals intravaginally inoculated with TK$^+$ virus (333 or DT1) developed symptomatic disease and were dead by day 8. In contrast, only 3 of 12 mice inoculated with TK$^-$ virus (333/TK$^-$ or DT2) exhibited genital symptoms ($P = .003$, Mann-Whitney U test), and none developed neurologic symptoms or died ($P < .01$, Fisher’s exact test).

Discussion

We believe this case of acyclovir resistance in a premature neonate is the first to be described. Resistance emerged after an initial course of acyclovir treatment and was associated with disseminated recurrent disease. This case is unusual for several
reasons. First, the resistant isolate was associated with an early recurrence of disseminated disease. Most neonatal recurrences are limited to skin, eye, and mucous membranes alone or occasionally in association with central nervous system disease [9]. The reasons for disseminated recurrence in this infant are unknown. It is likely that both a premature immune system and acyclovir resistance were contributing factors. However, we cannot exclude the possibility that the infant was infected by a mixed population of acyclovir-sensitive and -resistant HSV.

Second, the acyclovir resistance emerged after a 21-day course of acyclovir or shortly after reinitiation of acyclovir in a premature newborn. Most reported cases of acyclovir resistance have been in infants and children with congenital immunodeficiency or in immunocompetent full-term infants. One infant was receiving prolonged acyclovir prophylaxis after neonatal infection [10]. Of note, virus isolated from a subsequent recurrence had reverted to acyclovir-sensitive phenotype. The other case occurred in a full-term 10-day-old baby with infection of the larynx. However, whether the resistance occurred de novo or emerged after initiation of therapy could not be determined [11].

Third, the mutation found in this case was unusual. Although acyclovir resistance is usually mediated by mutations in the viral tk gene, as in this case, most described deletions or insertions occur in homopolymer runs of Gs and Cs or, rarely, As [12]. In this case, a deletion of thymidine at nt 927 resulted in early termination.

Results in our growth studies and mouse model suggest that the acyclovir-resistant strain is less virulent. However, this infant’s course suggests a possible disparity between in vitro findings and the magnitude of clinical disease observed. Several factors might contribute to this disparity. Most animal studies are conducted with adult immunocompetent mice. This neonate had an immature immune system, and the mouse model may not accurately reflect human neonatal disease. In a study that compared newborn and adult mice, corneal inoculation of TK− virus caused increased mortality and higher viral replication in trigeminal ganglia in newborn mice than in adult mice [13]. Moreover, human TK may functionally replace viral tk to a greater extent in vivo than observed in vitro or in animal models. Cellular TK compensates for lacking viral TK in multistep growth in BHK cells (this report) and in single-step growth in L-M cells (American Type Culture Collection, Rockville, MD) [14]. In addition, human tk functionally replaced HSV-1 tk for viral replication in mouse sensory ganglia and reactivation from latency following corneal inoculation [15].

In conclusion, we describe the rapid emergence of a clinically significant acyclovir-resistant HSV-2 isolate in a premature infant with perinatal disease. Acyclovir therapy appears to have selected the TK− isolate, since the initial and recurrent isolates had identical digest patterns. Although skin recurrences are common and are usually associated with acyclovir-sensitive virus, resistance should be considered in infants with disseminated recurrences or in those who do not respond appropriately to acyclovir therapy.

Acknowledgments

We are grateful to Li Jin Dong for providing primary cell cultures, Richard Roller for providing BHK(tk−) clone 100, Lawrence Stanley for providing 333(tk−) virus, Jung Guo and Barbara Hendrickson for assistance with the animal studies, and Xiao Chuan Zhou for excellent technical assistance in performing acyclovir-susceptibility assays.

References